CS may have GalNAc 4-O- or 6-O- sulphation and additionally GlcA 2-O-sulphation. CS from trachea is mainly GalNAc 4-O-sulphated while that from load bearing cartilages have higher levels of 6-O-sulphation.CS is widely used by those suffering osteoarthritis and a deeper understanding of CS structure/function relationships. However, available tools permit acquisition of compositional, but not sequence data, from CS, and it is likely that CS functional motifs will be embedded within domains of sequence.
Methods: We have used novel chondroitin C lyase depolymerisation methods allied with HPLC and novel Mass Spectrometry (MS) methods of oligosaccharide analysis to study CS composition and sequence. In addition intact CS chain structure has been studied by NMR spectroscopy.
Results: We have demonstrated that CS sulphation is non-random, that CS sulphation occurs in domains and that there are differences in CS composition and sequence between species. Data from shark cartilage show that GalNAc 4-O- and 6-O-sulphation occurs in blocks showing the domains of structure within a non-random CS chain. Shark CS contains 2-O-sulphated residues which are mostly found between residues with 4-O- and 6-O-sulphation in agreement with studies of the sulphotransferase activity. The 2-O-sulphation level increases with length, suggesting a preferential localisation towards the non-reducing end of the chain. Examination of tracheal CS from a range of mammals (e.g. human, bovine, porcine and ovine) shows that the composition and sequence of each differs. Further, we show that the CS from non-mammalian species e.g. chicken, is also unique. Many of these are used as sources of commercial CS and we therefore demonstrate that choice of species used to derive CS dictates the structure of the product. The level of 4-sulphation in bovine tracheal CS (*ca*. 70%) is significantly lower than that found in chicken CS (*ca*. 80%) and while the distribution of 4-sulphates throughout chicken CS is relatively uniform, this is not the case for bovine tracheal CS in which longer chains contain more 4-sulphated residues. These differences in composition and sequence will significantly change the profile of the CS and hence functionality. We have isolated defined CS oligosaccharides of up to 20-mer and demonstrated both purity and structure by NMR spectroscopy. These represent the largest pure defined oligo-saccharides isolated to-date and they represent a significant resource for further studies.
Conclusions: We have shown that digestion of CS with chondroitin C lyase allows the abundance of blocks of 4-O-sulphated GalNAc residues to be determined in an analytical method, suitable for acquiring sequence related data from CS chains to increase our understanding above that available from compositional analysis. CS structure impacts interactions and it will be important to use these techniques to characterise binding sites for protein binding partners. The localisation of sulphation was confirmed to be non random in local requirements and in the abundance along the chain length. These data also confirm that MS methods are appropriate for acquiring quantitative data on CS oligosaccharide abundance from heterogeneous mixtures. We also show that NMR spectroscopy can be used to acquire detailed structural data from intact CS chains.
These data show that the source tissue used for CS preparation can have a significant impact upon the composition, sequence and hence functionality of the material isolated.

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PRODUCTION OF TREFOIL FACTOR PEPTIDE 3 (TFF3) IN OSTEOARTHRITIC CARTILAGE

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Purpose: The trefoil factor family peptide 3 (TFF3, also known as intestinal trefoil factor) has been implicated in epithelial tissue restoration and is found associated mainly with mucosal epithelial surfaces. In the present investigation we addressed the question whether TFF3 has also a role in articular cartilage restoration.
Methods: Healthy and osteoarthritic human articular cartilage samples were compared with regard to expression and production of TFF3 by means of RT-PCR, Western-blotting and immunohistochemistry. TFF3 production in cultures of human primary chondrocytes as well as T/C-28a2 chondrocytes after incubation with tumor necrosis factor a (TNFa) and interleukin-1 (IL-1) was determined by immunostaining on glass cover slides and immunodot blot. Immunohistochemical analyses were performed to study the expression of Tff3 in osteoarthritic cartilage of STR/Ort mice.
Results: We found mRNA expression of human TFF3 in both human healthy and osteoarthritic cartilage, whereas production on the protein level was only visible in osteoarthritic cartilage. In the early stage of osteoarthritis (OA) TFF3 was detected in the cytoplasm and in the extracellular matrix while in late stages only intracellular immunostaining was observed. TNFa and IL-1-treated primary chondrocytes stained clearly with TFF3 antibody, whereas untreated cells were negative. Some T/C-28a2 cells revealed positive TFF3-staining without pretreatment. After TNFa administration the staining was increased; IL-1 had no effect. Immunodot blot analysis of culture supernatants from primary chondrocytes supported the finding of increased TFF3 secretion after administration of TNFa and IL-1. STR/Ort mice, which are genetically predisposed to develop OA-like lesions in the knee joint, demonstrated positive TFF3-staining of some but not all chondrocytes especially in early stages of OA.
Conclusions: These findings suggest a possible protective role of human TFF3 during OA, due to a possible control of matrix degrading enzymes (MMPs) or apoptosis, and encourage further investigations.

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EFFECTS OF CYTOKINE STIMULATION ON NOTCH SIGNALING IN ARTICULAR CHONDROCYTES

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Purpose: Osteoarthritis (OA) and rheumatoid arthritis (RA) are characterized by high expression of the proinflammatory cytokines interleukin-1β (IL-1β), and tumour necrosis factor alpha (TNF-α). Several studies have reported the involvement of the Notch signaling pathway in the patophysiology of RA. Several studies have shown that stimulation of synoviocytes from patients with RA with TNF-α induces expression of Notch1, 4, and Jagged2 as well as nuclear translocation of the intracellular do-

main of the Notch receptor (NICD). These results prompted us to study the effects of Notch signaling after cytokine stimulation of articular chondrocytes.
Methods: Gene expression of several Notch markers in human articular chondrocytes were studied after TNF-α and IL-1β (10 ng/ml) stimulation for 24h using real-time PCR.
Results: Stimulation with either TNF-α or IL-1β resulted in almost the same effects on expression of Notch markers. Gene expression of Notch3, Jagged1, and HES5 were significantly decreased after stimulation with TNF-α or IL-1β. Notch1 was significantly repressed after TNF-α stimulation while its expression after IL-1β treatment only tended to decrease. Transcription of Delta4 was significantly increased after IL-1β stimulation and the expression slightly increased by TNF-α treatment.
Conclusions: This study shows that markers for the Notch signaling pathway in chondrocytes are affected by cytokine stimulation. Synoviocytes and chondrocytes respond differently to cytokine stimulation with regard to Notch signalling.

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INHIBITION OF MITOCHONDRIAL RESPIRATORY CHAIN ACTIVATES CICLOXYGENASE 2 PROTEIN EXPRESSION AND PROSTAGLANDIN 2 SYNTHESIS IN OSTEOARTHRITIC CHONDROCYTES

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Purpose: In this study we investigated whether inhibition of mitochondrial respiratory chain (MRC) is able to modulate the inflammatory response in human osteoarthritic (OA) chondrocytes and the implication of stress inducing signals in this process.
Methods: Human OA chondrocytes were isolated from cartilage obtained from patients undergoing joint replacement. Rotenone, 3-nitropropionic acid (NPA), antimycin A, sodium azide and oligomycin were employed to inhibit complex I, II, III, IV or V of MRC, respectively. Flow cytometry was used to know the COX-1 and COX-2 protein expression as well as reactive oxygen species (ROS) liberation. PGE_2 production was evaluated by ELISA. NF-κB expression was quantified with a transcription factor assay. To demostrate the role of ROS, Ca^{2+} and NF-κB in COX-2 expression induced by inhibition of MRC, N-acetyl cysteine 2mM (a ROS inhibitor), Ruthenium Red 1 μM (an inhibitor of mitochondrial Ca^{2+} exhange), and BAY 117085 1μM (an inhibitor of NF-κB activation) were used.
Results: Firstly, we assesed the production of the inflammatory mediator PGE_2 in OA cells in the presence of rotenone (10-50 μg/ml), NPA (0.5-10 mM), antimycin A (20-60 μg/ml), sodium azide (2-25 mM) and oligomycin (5-100μg/ml). IL-1β (5 ng/ml) was employed as positive control. After 48 h of stimulation, only antimycin A and oligomycin increased PGE_2 (basal, 15.61±1.86; antimycin A, 96.90±12.72 and oligomycin, 64.03±20.29 pg/50.000cells). Secondly, we studied the percentage of cells that expressed COX-1 or COX-2 protein in the presence of all MRC inhibitors. Again, the inhibition of complex III and V showed an increase in COX-2 protein. After 5 h of stimulation, antimycin A produced a 32.24±4.68% of positive cells and oligomycin a 28.89±4.13% respect to basal (12.91±1.96). This expression was maintained after 18 h of stimulation. COX-1 didn't show any modulation. Thirdly, as mitochondrial complex activity downregulation could be by ROS or calcium exchange, we studied whether these mechanisms contributed to the increase of COX-2 expression and PGE_2 production. Notably, the percentages of cell population that produced H_2O_2 after treatment with antimycin A and oligomycin at 5 minutes were 63.11±6.03% and 58.35±6.51%, respectively vs basal (36.47±9.99%). Pretreatment for 2 h with N-acetyl cysteine (NAC) 2mM (a ROS inhibitor), or Ruthenium Red (RR) 1μM (an inhibitor of mitochondrial calcium exchange) diminished the effect of antimycin in the COX-2 expression up to 22.82% and 18.27%, respectively. Values for oligomycin decreased up to 47.29% and 47.68%. As NF-κB is one of the transcription factors implicated in COX-2 expression, we examined if it's required for the increase of COX-2 protein induced by MRC inhibitors. Our results showed that a NF-κB inhibitor, BAY 117085 (1 μM), reduced in a 17.26% the effect of antimycin A and a 36.97% the effect of oligomycin in the expression of COX-2 protein.
Conclusions: These results show that the inhibition of MRC complex III and V induces an inflammatory response in human OA chondrocytes, that could be mediated by ROS, mitochondrial calcium exchange and NF-κB activation.

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ELECTRON MICROSCOPIC STEREOLOGY IN THE ANALYSIS OF CARTILAGE COLLAGEN FIBRIL NETWORK OF TRANSGENIC MICE

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Purpose: To investigate by electron microscopic stereology the properties of cartilage collagen fibril network in newborn transgenic mice. The gene-mutated mice harbored transgenes targeted to affect the structure or assembly of the collagen fibrils in murine cartilage.
Methods: Cartilage of murine tibial growth plate or nasal septum were investigated by EM stereological technique using isotropic, uniform random (IUR) sampling with isector, and indirect estimation of stereological parameters, i.e. volume fraction (VV) and surface density (SV), using average fibril diameter and collagen length density (LV) (Langsjo et al. 1999 and 2002). Five types of genetically mutated mice were investigated and compared to their wild-type (wt) littermates. The following mouse lines were investigated: (i) mice with one active murine Col2a1 gene and two copies of human COL2A1 transgene with Arg519Cys mutation (M+/-H) (Li et al. 1995, Arita et al. 2002), (ii) mice with no active murine Col2a1 gene alleles and two copies of the human COL2A1 transgene with Arg519Cys mutation (M-/-H) (Li et al. 1995, Arita et al. 2002), (iii) mice harboring no active murine Col2a1 gene alleles and four copies of human COL2A1 transgene with Arg519Cys mutation (M-/-HH) (Li et al. 1995, Arita et al. 2002), (iv) mice harboring a copy of human COL2A1 transgene with deleted exons 16-27 and two wild-type Col2a1 alleles (M+/+COL2A1-Del) (Helminen et al. 1993), and (v) mice with inactive alleles of procollagen N-proteinase (PNP-KO) (Li et al. 2001). N-proteinase cleaves off the N-propeptides of procollagen monomers making the collagen molecule able to incorporate within the fibril.
Results: In all newborn (1 to 2 days old) mice carrying the COL2A1 transgene with Arg519Cys mutation (M+/-H, M-/-H, M-/-HH) the growth plate collagen fibrils were thinner (diam. 15 to 16 nm) than in wt mice (diam. 18 nm) and showed reduced volume fraction (%) of the fibril network ($p < 0.05$ - 0.001). On the other hand, in M+/+COL2A1-Del mice, the collagen fibril thickness remained unaltered but the volume fraction of collagen was reduced ($p < 0.05$). In PNP-KO mice, fibril thickness and the volume fraction of collagen did not differ from the wt mice.
Conclusions: The EM stereological technique proved to be efficient in revealing differences in the collagen fibril thickness and the collagen volume fraction in newborn mice harboring mutations which affected structure and assembly of collagen fibrils. This made possible early detection of the altered phenotype.